

AD _____

Award Number: DAMD17-01-1-0707

TITLE: Steroid Hormones in NF1 Tumorigenesis

PRINCIPAL INVESTIGATOR: Margaret R. Wallace, Ph.D.
David Muir, Ph.D.
Martha Campbell-Thompson, DVM, Ph.D.

CONTRACTING ORGANIZATION: University of Florida
Gainesville, Florida 32611-0266

REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030313 161

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 01 - 31 Jul 02)	
4. TITLE AND SUBTITLE Steroid Hormones in NF1 Tumorigenesis			5. FUNDING NUMBERS DAMD17-01-1-0707	
6. AUTHOR(S) Margaret R. Wallace, Ph.D. David Muir, Ph.D. Martha Campbell-Thompson, DVM, Ph.D.			8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Florida Gainesville, Florida 32611-0266 E-Mail: peggyw@ufl.edu				
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) NF1 is characterized by benign Schwann cell tumors called neurofibromas; complex forms can become malignant. Steroid hormones are implicated in cancer, but little is known about these in NF1. The growth response of NF1 tumor Schwann cells to estrogen and progesterone will be characterized. The hypothesis is that human neurofibroma (and/or MPNST) Schwann cells have increased hormone response compared to normal Schwann cells, leading to tumor growth. Specific Aim 1 will determine steroid hormone receptor expression in human normal, NF1 neurofibroma and MPNST Schwann cells. Real-time PCR has shown low levels of these receptor transcripts in untreated normal and tumor cells, with very little change in the tumors (much less than 2-fold). Interestingly, 2 tumors from one patient had very similar results. Initial protein experiments suggest that the basal receptor levels are very low. Specific Aim 2 will test the in vitro response of tumor cells to hormones. The proliferation assay conditions have been refined and applied to normal human and rat Schwann cells, as well as an MPNST line so far (which showed no proliferative response). Specific Aim 3 involves in vivo hormone response of human tumor cells explanted into a mouse nerve; these experiments have just begun.				
14. SUBJECT TERMS NF1, neurofibroma, Schwann cell, estrogen, progesterone			15. NUMBER OF PAGES 9	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	page 1
SF 298.....	page 2
Introduction.....	page 4
Body.....	page 5
Key Research Accomplishments.....	page 8
Reportable Outcomes.....	page 8
Conclusions.....	page 8
References.....	page 9
Appendices.....	page 9

INTRODUCTION

Clinical literature suggests that steroid hormones may play a role in NF1 since neurofibroma growth shows some parallels with hormonal changes (Dugoff and Sujansky, 1996). For example, many patients develop neurofibromas at puberty, pregnancy often increases tumor size/number, women with NF1 may have a higher risk of malignancy and a higher neurofibroma burden, and neurofibroma development often slows in older adults. The steroid hormone field is being intensely researched in many types of cancer, but virtually nothing is known about these pathways in normal or NF1-tumor derived Schwann cells. Schwann cells comprise the bulk of these tumors, in which a large percentage are somatically mutated and clonally expanded (Rutkowski et al., 2000; Wallace et al., 2000; Serra et al., 2000).

Our goal is to characterize the growth responses of human normal and NF1 tumor Schwann cells (neurofibromin-negative) to steroid hormones, focusing on estrogen and progesterone. The hypothesis is that human neurofibroma (and MPNST) Schwann cells have increased hormone responsiveness compared to normal Schwann cells, leading to tumor growth. The role of the hormone receptors is a growing topic in cancer research, but little is known about these in normal or NF1 Schwann cells.

In Specific Aim 1, we will determine steroid hormone receptor expression in human normal, NF1 neurofibroma, and NF1 MPNST Schwann cells, pre- and post-hormone treatment by studying archived material and cell culture materials. We have a number of NF1 tumor-derived enriched Schwann cell cultures that have been found to lack neurofibromin and will be the basis for this work (Muir et al., 2001). In Specific Aim 2, we will test *in vitro* responses (proliferation and cell survival) of cultured human Schwann cells from normal nerve, NF1 neurofibromas and NF1 MPNSTs, to estrogen and progesterone and their antagonists. Specific Aim 3 will test *in vivo* responses (proliferation and survival) of NF1-derived tumor Schwann cell lines to estrogen and progesterone, through a xenoplant model in *Nf1/scid* mice.

Regardless of the data that emerge, whether showing positive or negative or mixed response, this will be an important step in scientific knowledge about the role of these hormones in NF1. This will have impact on patients who are candidates for hormone therapies, and may lead to new targets for anti-tumor therapies.

Task 1. To characterize the steroid hormone receptor profile in 24 normal and NF1 tumor Schwann cell cultures pre- and post-hormone treatment (months 1-30).

Progress on Task 1a: "To measure initial expression, RNA from cells grown under normal culture conditions will undergo semi-quantitative RT-PCR to evaluate transcript levels of steroid receptors, a quantitation control, and selected downstream targets. Primary tumor samples corresponding to cultures will be surveyed as well. Immunohistochemistry of primary tumor sections will also be done with for receptors showing transcripts (months 1-12)."

Thus far, 10 tumor RNA samples (3 primary, 7 cultures grown under standard conditions, and 2 normal Schwann cell cultures) have been analyzed for estrogen receptor alpha (ERa), estrogen receptor beta (ERb), progesterone receptor (PR), and androgen receptor (AR) expression through use of real-time PCR (Roche Light Cycler), normalized with respect to the HPRT housekeeping gene and one of the normal cell lines. The studies were done in triplicate and averaged, to ensure consistency of data. The table below shows the results, where blanks indicate PCR reactions that did not work. In summary, overall there were no dramatic changes in expression compared to normal. For ERa, about half of samples showed slight decrease and half showed slight increase. ERa is the receptor though to be most involved in positive proliferation signals in other tissues, such as breast. Of possible interest, the lowest ERa signal was in a sarcoma (MPNST) line. The same observation was seen for ERb, where about half of samples had minor increases and others had slight decreases. ER beta's roles are still being discovered in other tissues, and it appears it can have complex functions. Progesterone receptor seemed to show uniform increases in all samples, with one sample having twice the normal amount of transcript compared to normal, the largest difference seen in any of the work thus far. AR also showed a mixed pattern. None of the changes correlated with gender of the sample. These preliminary data indicate that the expression differences in primary or cultured cells from NF1 tumors, without hormonal treatment, are very minor, almost all less than 2-fold. More samples will be done to add to these data, including RNA from normal female Schwann cells recently obtained. Also, we are attempting to immunostain archived primary tumor sections to correlate with the RT-PCR data. However, it is clear that these receptors are expressed at very low levels, and the first few immunohistochemistry assays for PR and ERa appeared negative in the NF1 samples despite the positive controls being quite strong, and despite use of fresh tissue blocks and sections. Thus, a protein-based approach may not be sensitive enough to measure these molecules.

Table 1: Light Cycler real-time PCR data. The first 3 samples are primary RNAs, the next 7 are from tumor Schwann cell cultures, and the last 2 are from normal (non-NF1) cultures, all treated under standard culture conditions. Results are shown in values normalized to the normal pn97.3 data.

Sample	gender	Tumor type	ER alpha	ER beta	PR	AR
328T7	F	Dermal	1.0964	1.1329	2.0710	1.3168
362T	M	Plexiform		1.2514	1.5886	1.4450
459T	M	MPNST	1.0545	1.0619	1.2059	0.9314
cNF96.5g	F	Dermal	0.9362	0.8681	1.1972	0.9818
cNF97.2a	F	Dermal	1.0373	1.0869	1.1915	1.0746
cNF98.4a	F	Dermal	0.9224	0.9679	1.1761	0.9541
cNF98.4d	F	Dermal	0.9385	0.9499	1.1691	0.9666
pNF95.11b	M	plexiform	0.8062	0.9975	1.0562	0.9659
sNF96.2	M	MPNST		1.2157	1.6840	
sNF94.3	F	MPNST	0.7166	0.9595	1.2419	1.1800
pn97.3	M	normal	1	1	1	1
pn97.4	M	normal	0.9636	1.0209	1.1253	0.8297

Progress on Task 1b: "Cultures treated in Task 2 will be analyzed with RT-PCR as above. (months 3-30)."

As above, the same studies will be performed on the treated Schwann cell cultures. These treatments (as part of Task 2) are just now underway and so the RNA samples are being accumulated.

Progress on Task 1c: "Cultures positive for receptors by RT-PCR (pre- and/or post-treatment) will be analyzed by Western blot and immunofluorescence (months 3-30)."

Conditions necessary for Western blot and immunofluorescence are being worked out now. As indicated above from preliminary immunohistochemistry, it is possible that protein-level analyses may not be sensitive enough for these molecules, although the resources (fixed chamber slides from cultured cells, protein extracts from treated cultures) are being accumulated, so that this work will be attempted once sufficient samples are in hand for periodic batching.

Task 2. To measure cultures' proliferative and apoptotic response to hormone and SERM/antagonist treatment (months 1-30).

Progress on Task 2a: "Cultures will be treated with hormones/SERMs separately and proliferative response measured with BrdU assay, and cell survival measured by counting and TUNEL assay. (months 3-18)."

The BrdU proliferation assay conditions and system were established for normal human Schwann cells, which showed that 1% serum is ideal for replicating baseline proliferation rates. Rat Schwann cells and MCF-7 (breast cancer) cells were next used as preliminary controls, with the rat Schwann cells showing no response to estradiol or ICI182780 (the estradiol antagonist). The MCF-7 cells showed strong proliferation to estradiol as expected, and inhibition with addition of the antagonist.

The proliferation assays of tumor samples have just gotten underway. The first culture studied was sNF96.2 (an MPNST), with estradiol and progesterone concentrations ranging from 0-100 nM, ICI182780 at 1 uM, and controls and replicates employed as described in the proposal. No statistically significant change in proliferation rate was seen under any treatment, using the chi-square test. RNA and protein were saved at time points 0, 3 and 7 days, for the analyses above. Study of other cultures is now underway and should progress well. The first attempt at the TUNEL apoptosis assay, using the same cells and a kit from Promega, failed due to tissue culture problems. However, this has been remedied and should progress in step with the proliferation assays.

Progress on Task 2b: "Cultures showing a response will undergo dose response testing, and assays in (a) will be repeated using combinations of appropriate hormones/SERMs/antagonists. (months 12-30)."

Not yet begun.

Progress on Task 2c: "Statistical analysis (months 24-36)."

Not yet begun, other than analysis of individual culture responses.

Task 3. To assay for *in vivo* proliferative or survival response of tumor cells to estrogen and progesterone. (months 12-36).

Progress on Task 3a: "Select 6 tumor and 2 normal Schwann cell cultures (in part based on *in vitro* results)."

Since *in vitro* results are not yet complete, we have not fully selected all cultures for the *in vivo* work, but nonetheless will begin with the MPNST studies since we have so few of those cultures that these would obviously be chosen. Selection of the subsequent cultures (plexiform and dermal) will be based on *in vitro* data and real-time PCR data.

Progress on Task 3b: "For each culture, sterilize 15 *scid* mice heterozygous for an *Nf1* mutation, inject cells, and treat mice with estrogen, progesterone, or placebo."

The colony of *Nf1/scid* mice is doing very well and should provide an adequate number of female mice on a fairly constant basis. The postdoctoral fellow hired to do this

work (arrived in April) has perfected the surgery and hormone pellet implant techniques, and has just begun with our first culture, an MPNST. 6 mice have been treated thus far, with cells injected into both sciatic nerves. Thus, this task is slightly ahead of schedule.

Progress on Task 3c: "Perform proliferation assay (Ki67 staining) and TUNEL assay on xenopplants by analysis of tissue section, and statistical analysis."

Not yet underway (treated animals must be 2 months post-op prior to sacrifice and study of treated nerves).

KEY RESEARCH ACCOMPLISHMENTS

- Determined that the basal steroid receptor levels in normal and NF1 neurofibroma Schwann cells are very low (supported by real-time PCR and immunohistochemistry data), and that the tumor cells do not show dramatic changes in the levels of these receptor transcripts.
- Perfected most of the in vitro and in vivo techniques for Aims 2 and 3.

REPORTABLE OUTCOMES

There are no reportable outcomes yet, this being the first year. And in fact, there was a delay in beginning this work, due to administrative miscommunications at my university—the funds for this work were not released to me until November, and so the data actually represent 8-9 months effort rather than 12.

CONCLUSIONS:

Although still preliminary, the data currently suggest that steroid hormone receptor profiles in normal and NF1 tumor Schwann cells are low and may not be very different, and that any changes vary from patient to patient (some up, some down). In Table 1, two of the dermal cultures are from tumors from the same patient (surgery at the same time), and it is interesting that their data are very similar. This suggests that there may be little variation between the same types of tumors in an individual, which is very important for future therapies; delivery of a systemic drug to affect steroid signaling might not be a good strategy if patients' tumors have variable receptor profiles and thus might respond differently. We will analyze a few more tumor sets from the same patients to see if this trend continues. Also, the work to study the response of these cells to hormones and antagonists is now underway. Even though there appears to be generally low expression, and small basal receptor level differences in tumors, it is entirely possible that these small changes may be functionally very significant under the influence of hormones—our work will test this theory using in vitro and in vivo approaches. We have shown that one MPNST (from a male) does not respond proliferatively in cell culture to steroid hormones; it will be interesting to see if that pattern is also true of a female MPNST line now under study, as well as the other cell cultures to be done this year. We are striving toward the first laboratory-based NF1 data about human cell steroid hormone responses, which will

provide a rational basis for medical decisions about hormone therapies in NF1 patients (e.g. hormone replacement, birth control pills, cancer treatments—will neurofibromas be aggravated by these therapies?), and also test whether the steroid hormone system is a good target for specific tumor growth control.

REFERENCES:

Dugoff L, Sujansky E. Neurofibromatosis type 1 and pregnancy. *Am J Med Genet* 66:7-10, 1996.

Muir D, Neubauer D, Lim IT, Yachnis AT, Wallace MR. Tumorigenic properties of neurofibroma Schwann cells. *Am J Pathol* 58:501-513, 2001.

Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E. Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. *Hum Mol Genet* 9:1059-1066, 2000.

Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, Lenard H-G, Lazaro C. Schwann cells harbor the somatic NF1 mutation in neurofibromas" evidence of two different Schwann cell subpopulations. *Hum Mol Genet* 9:3055-3064, 2000.

Wallace MR, Rasmussen SA, Lim IT, Gray BA, Zori RT, Muir D. Culture of cytogenetically abnormal Schwann cells from benign and malignant NF1 tumors. *Genes Chrom Cancer* 27:117-123, 2000.

APPENDICES: None